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III. AGAR ANAPHYLATOXIN: RABBIT SERUM

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SUMMARY

Bordet[1] stated that diverse fresh sera behave like that of the guinea-pig on contact with agar. It is to be presumed that this statement includes rabbit serum, but of that there is no certainty. Tchernoroutzky[2] specifically stated that the sera of rabbit, horse, and man, when treated with agar, become about as toxic as the serum of adult guinea-pigs (2 to 3 c.c. per 100 gm.). On the other hand, Haren[3] reached the conclusion, based on insufficient data, that agar anaphylatoxin is toxic only for the homologous animal. In his tests he employed treated rabbit serum, which in dose of 2 to 3 c.c. produced no effect in guinea-pigs. It will be shown that his deductions were wrong.

There is no doubt but that rabbit serum, in general, is more difficult to toxify than is that of the guinea-pig. The least difficult in that respect is rat serum. The work with rabbit serum was included in this study for the reason that it is possible to obtain a large amount with the least outlay of time and expense. This fact to a large extent counterbalanced the difficulty or slowness in producing anaphylatoxin.

In a good portion of this work, the sera of fasting rabbits (2 to 24 hours) were employed in the belief that fasting gave a more uniform serum and made it more toxifiable. A brief fast for a few hours seemed to be better than one of a day or more. But even under these conditions, great variation in the ease or intensity of poison-production was encountered. A cloudy or lipoidal serum often gave better results than one which was water-clear. It is probable that the individual variation in the rabbits is a more important factor than it is with the guinea-pig or rat.

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[1] Compt. rend. Soc. de biol., 1913, 74, p. 227.
[2] Ibid., p. 1213.
[3] Ztschr. f. Immunitätsf., 1914, 20, p. 676.

The uncertainty of results with rabbit serum proved often to be most disconcerting. Thus, an endeavor to repeat and extend the experiments given in Tables 35 and 42, made a year later, led to uniformly negative results. This striking difference could be accounted for in no other way than that the diet of the laboratory animals, on account of market conditions, was less varied than it had been before. The prohibitive cost of vegetables necessitated an almost exclusive hay diet, whereas in the previous year lettuce, cabbage, beets, carrots, bread, oats, etc., were freely given along with but little hay. A series of experiments, extending over more than a month, was made to locate the difficulty, but with no marked success. On a cabbage diet, the rabbits gave sera more promptly toxifiable with agar than on other food; but even this result was not dependable. A bread diet for a short time, likewise, appeared to favorably influence the serum. On the other hand, exclusive feeding of oats or carrots did not appear to make the serum more toxifiable. The most that can be said is that a daily varied diet is better than a single monotonous one.

No effort was made to reduce the lethal dose below 3 c.c., but it is quite possible that this can be done since the dose mentioned very often caused acute death in less than 3 minutes. The speed of poison-production, as in the case of guinea-pig and rat serum, is rapid, for frequently this dose proved to be fatal after an incubation of but 15 or 30 minutes. Failures, however, were more frequent, and with many rabbit sera, tho tests were made every 15 minutes, no fatal shock was obtainable until after 1½ to 2 hours, and sometimes even no result was obtained after incubation for twice that length of time.

The difficulty in toxifying a rabbit serum so that 3 c.c. represent more than 1 lethal dose accounts in part for the variable results in guinea-pigs. It will be shown at the end of this paper that the resistance of the guinea-pig is by no means uniform and this fact becomes more prominent the weaker the anaphylatoxin. Hence, the irregularity in results and the relatively few fatal shocks.

It must be borne in mind that normal untreated rabbit serum may produce acute death in dose of 3 c.c. This is especially true when it has been kept in contact with the spontaneous clot for some time, or when bead-defibrination is resorted to. A full discussion of this question will be found in Part VIII, which deals with the primary toxicity of normal sera. It should be stated here that the sera for this work with agar were from perfectly normal rabbits and were always obtained by rod defibrination, a method which gives the least toxic serum; the acutely lethal dose of such sera is usually about 6 c.c.

When the physical condition of the rabbit is not taken into consideration, it is possible to obtain a serum which will have a greater initial toxicity. This appears to be the only explanation for some of the highly toxic rabbit sera obtained by Mita and Ito. Incidentally, it may be stated that we have found the serum of a cachectic rabbit to be acutely fatal in dose of 0.05 c.c. per 200 gm. of guinea-pig, a toxicity 120 times greater than that of the ordinary normal serum. It may be assumed that the matrix of the poison is increased in the sick animal, but it is equally reasonable to believe that this toxicity is due to a decrease in the amount of the antagonistic or calyptic substances present in the normal plasma.

It will be frequently found that a rabbit anaphylatoxin will produce a delayed death, one or more hours after injection. Such effects are to be ascribed to the primary toxicity of the serum, rather than to a fresh yield of anaphylatoxin. These facts indicate the need of parallel control tests with the same pooled serum, for without such careful controls an experiment can have but little value.

As regards the technic, it may be stated that the rabbits were always bled from the carotid, the blood being drawn directly into sterile carotid pipets, which have been described in Part I. The blood was immediately defibrinated with the glass rod, and then transferred to sterile special tubes and centrifugated at 8000 revolutions for from 5 to 30 minutes. The perfectly clear serum was usually employed at once, that is to say within about an hour after the start of bleeding. It can be toxified, however, even after it has been kept for 24 hours. The preparation of the agar, and its mode of employment were the same as given in Part II.

EXPERIMENTS WITH AGAR SOL, 50 C.

The first experiments with rabbit serum were made under conditions similar to those given in Table 19. The mixtures of 50 C. sol and serum, previously warmed to 37 C. (0.25 : 1), were thoroughly shaken for 1 minute, then centrifugated at 3000 revolutions for 5 minutes, either at once or after incubation at 37 C. for 2½, 5, 10, and 15 minutes. The centrifugated serum was then placed at 37 C. for 2 hours, after which it was tested. In one set of experiments, the equivalent of 3 c.c. of serum was injected; in another set an injection of 4 c.c. was used. The effects in both sets were slight or nil.

In another experiment, the sol-serum mixture being in the ratio of 0.025 : 1, and the incubation of the five portions before centrifugation being the same as given, of 5 tests with 4 c.c., 4 caused delayed deaths after some hours. Such protracted deaths show little or no increase over the primary toxicity of the serum employed. It will be seen, therefore, that the results by this method were less favorable than in the corresponding experiments with guinea-pig serum (Table 15).

EXPERIMENTS WITH AGAR GEL, 0 C.

The experimental conditions here corresponded with those for guinea-pig serum as given in Table 22. The iced gel was added to 4 parts of serum, previously cooled to 0 C.; the mixtures were thoroughly shaken for 2 minutes, after which they were placed at 37 C. for a variable time. They were then centrifugated at 3000 revolutions for 8 minutes; tests were made at once and after incubation for the time stated in Table 32. Three different sera were employed for these experiments; one was used for Expers. 1 and 2; another for Expers. 3, 4, and 5, and a third was used for Expers. 6 and 7. The protracted death in No. 4 must be ascribed to the primary toxicity of the serum used. The acute deaths in Nos. 9 and 11 are clearly due to anaphylatoxin.

TABLE 32

ACTION OF AGAR GEL (0 C.) ON RABBIT SERUM (0 C.) (RATIO 0.25:1. MIXTURE KEPT AT 37 C. THEN CENTRIFUGATED AND REINCUBATED)

Exper.	Guinea-Pig		Serum			Result
	No.	Weight	B. C. at 37 C.	A. C. at 37 C. (hr.)	c.c. (intravenously)	
I	1	175		3	4	Moderate shock
	2	170		19	"	Slight
II	3	178	10′	3	"	"
	4	171	10′	19	"	5½ hr.
III	5	202	30′		"	Very slight
	6	192	30′	4	"	Slight
IV	7	171	1 hr.		"	Very slight
	8	180	1 hr.	4	"	Slight
V	9	188	2 hr.		"	2′30″
	10	185	"	4	3	Slight
VI	11	171	"		4	4′50″
	12	175	"	7	"	Slight
VII	13	200	—	2	"	Slight

EXPERIMENTS WITH SOL-GEL AGAR

The sol-gel mixtures were prepared as described in connection with the work on guinea-pig serum. The mixtures were placed in cracked ice for 3 hours, after which a portion was removed and centrifugated at 3000 revolutions for 4 minutes and injected; the balance was placed at 37 C. and similarly tested every 15 or 30 minutes up to 3½ hours. The equivalent of 3 c.c. was injected in all tests. Only Mixtures 3 to 8 (Table 23) were made with rabbit serum. Mixtures 1 and 2 were not tried because of their failure to toxify guinea-pig serum.

One series of tests was made with each mixture in order to ascertain their relative toxifying values. The results of these tests will be briefly summarized. Mixtures 3 and 4 were made with one serum, 5 and 6 with another serum, and 7 and 8 with still another.

Mixture 3.—Fair shocks were obtained after incubation for ¼, 2, and 3½ hours; the test made at 3 hours killed in 3 minutes 20 seconds. It is evident that this mixture, having an agar-serum ratio of 1 : 40000, is capable of toxifying rabbit serum.

Mixture 4.—The test made after incubation for one-half hour killed in 8 minutes 20 seconds, while that at 3½ hours was likewise fatal in 3 minutes 30 seconds. A severe shock was obtained in the test made at 2 hours; the results of the other tests were slight.

Mixture 5.—The tests with this and the following mixture were made at intervals of 15 minutes up to 1¼ hours. The test made at one-half hour gave a good shock; those at 1 and 1¼ hours killed in 6 minutes and 3 minutes 10 seconds, respectively.

Mixture 6.—The immediate test gave a severe shock while that at one-quarter hour was moderate. The tests at ½, ¾, and 1 hour killed in 3 minutes, 3 minutes 35 seconds, and 3 minutes 45 seconds, respectively. This mixture, which represents an agar-serum ratio of 1 : 2000, was apparently the most active.

Mixture 7.—The tests made at once, and at 1½, and 2 hours gave fair shocks; those at ¾ and at 3½ hours killed in 13 and 4 minutes, respectively. The other tests gave but slight effects.

Mixture 8.—The agar-serum ratio in this mixture was 1 : 400, and this fact may have something to do with the protracted deaths. The serum used was of the same pool as that of Mixture 7, with which the deaths were acute or nearly so. In this set, the immediate test killed in 85 minutes, while that at one-half hour took 25 minutes. The other tests made up to 3½ hours had but slight effects.

A summary of these tests is given in Table 33. It will be seen that of 52 tests 12, or 23%, were fatal. The conclusion to be drawn is that rabbit serum can be toxified by the sol-gel treatment so that 3 c.c. will produce a typical acutely fatal shock. The optimal mixture appears to be No. 6. An excess of agar seems to be unfavorable.

TABLE 33

SUMMARY OF SOL-GEL RABBIT-SERUM MIXTURES, SHOWING DISTRIBUTION OF FATAL SHOCKS (MIXTURES KEPT AT 0 C. FOR 3 HR., THEN AT 37 C.; DOSE 3 C.C. SERUM)

Mixture	Ratio	Time When Tested in Hours										
		0	¼	½	¾	1	1¼	1½	2	2½	3	3½
3	0.005:1	—	—	—	—	—		—	—	—	+	—
4	0.025:1	—	—	+	—	—		—	—	—	—	+
5	0.05 :1	—	—	—	—	+	+					
6	0.1 :1	—	—	+	+	+	—					
7	0.25 :1	—	—	—	+	—		—	—	—	—	+
8	0.5 :1	+	—	+	—	—		—	—	—	—	—

— = nonfatal effect
+ = death

The results shown in the preceding table must not be interpreted as constant or fixed for a given mixture. They will necessarily vary with the individual serum and in addition with the chance resistance of the guinea-pigs, as well as slight variations in the technic. Since Mixture 6 seemed to give the best results, it was used in nearly all the subsequent work and hence a number of these experiments are available for comparison. A summary of these experiments is given in Table 34, which shows only the fatal shocks. In all, the sol-gel mixtures were incubated till the end, only a small portion, the equivalent of 3 c.c. of serum, being removed, centrifugated, and tested at each period.

TABLE 34

SUMMARY OF EARLY TESTS WITH SOL-GEL RABBIT-SERUM MIXTURE 6, SHOWING DISTRIBUTION OF FATAL SHOCKS

Exper.	Time When Tested in Hours								
	0	¼	½	¾	1	1¼	1½	1¾	2
1	—	—	+	+	+	—			
2	—	—	—	—	—				
3	—	—	—	+	+	—			
4	+	—	—	—	—	—			
5	—	—	—	+	—	—	+	—	
6	—	—	+	—	—	—	+	—	+
7	—	—	—	—	+	—	—	—	—
8	+	—	+	—	—	—	—	—	—
9	—	—	—	—	—	—	+		
10	—	+	—	—					
11	—	—	+						
12	—	+							
13	—	+							

— = nonfatal effect
+ = death

It will be seen that only 1 of the 13 experiments tabulated failed to produce a fatal shock. Similar experiments made a year later met with a considerably larger number of failures, the only apparent reason, as has already been pointed out, being a difference in diet. It is to be noted that the immediate tests made in Experiments 4 and 8 resulted in acutely fatal shocks; also that in 3 of the experiments the sera became fatally toxic after incubation for only 15 minutes. It will be seen further that of 76 tests 19, or 25%, proved fatal.

CENTRIFUGATION OF SOL-GEL MIXTURES

It has been shown that mixtures of agar and guinea-pig serum, when centrifugated at high speed immediately after icing, do not become toxic on subsequent incubation at 37 C. A similar experiment with rabbit serum gave likewise a negative result. Inasmuch as this experiment to a certain extent serves as a control for those which follow, it merits being given in a condensed form.

A sol-gel mixture, No. 6, consisting of 4.2 c.c. of sol and 42 c.c. of serum, both at 37 C., after vigorous shaking for 1 minute was placed in cracked ice for 2 hours. A small portion, representing 3 c.c. of serum, was then removed, centrifugated, and injected; the balance was centrifugated at 8000 revolutions for 1 hour, after which the clear treated serum was placed at 37 C. and tested at intervals of 15 minutes, up to 2 hours. The 10 consecutive tests made with this treated serum were uniformly negative, or at most had very slight effects.

This experiment, like that of the corresponding one with guinea-pig serum, shows that mere icing of the agar-serum mixture does not create a sufficient disturbance to toxify the serum. Were the reaction one of simple adsorption of an inhibiting substance, it would be reasonable to expect such adsorption to occur at a low temperature, to a moderate extent at least, and such treated serum after removal of the agar should give evidence of increased toxicity. Such, however, is not the case.

Incubation of the agar-serum mixture at about 37 C. is necessary to poison-production. After the reaction is once induced, the agar can be removed completely from the mixture and such treated serum on incubation will maintain its toxicity for some time, as will be seen from the following experiment.

A sol-gel mixture, No. 6, consisting of 6 c.c. of sol and 60 c.c. of serum was treated in the same way as the preceding, except that after it had been iced 2 hours, and a portion removed for an immediate test (No. 1), the balance was placed at 37 C. for 15 minutes; then a portion was removed, centrifugated, and tested (No. 2) to see if the mixture had become toxic. This being the case, the entire mixture was immediately centrifugated at 8000 revolutions for 60 minutes; the resulting clear serum was tested at once, and the balance placed at 37 C. and tested at intervals of 15 minutes. The equivalent of 3 c.c. was used for all the injections.

A comparison of the results of this experiment, Table 35 and Chart 6, with those of a similar experiment with guinea-pig serum (Table 27) shows a marked agreement. In both, the toxicity persists after removal of the agar. The speed of poison-production is indicated by the fact that incubation for 15 minutes or less suffices to toxify. The apparent fluctuation in the toxicity must be ascribed to variations in the resistance of the recipients.

TABLE 35

CENTRIFUGATION AT 8000 R.P.M. APPLIED AFTER INCUBATION AT 37 C. FOR 15 MINUTES (SOL-GEL MIXTURE 6, RATIO 0.1:1)

Guinea-Pig		Serum			Result
No.	Weight	B. C. at 37 C. (min.)	A. C. at 37 C. (hr.)	c.c. (intravenously)	
1	175	0		3	Very slight
2	170	15		"	3′15″
3	197		0	"	Moderate
4	171		¼	"	3′
5	188		½	"	Slight
6	172		¾	"	2′45″
7	205		1	"	Fair
8	176		1¼	"	5′
9	179		1½	"	Slight
10	201		1¾	"	Very slight
11	176		2	"	Severe
12	174		2¼	"	3′
13	193		2½	"	12′
14	188		2¾	"	Very slight
15	199		3	"	Slight

Inasmuch as it would be of interest to know how long this toxicity would persist in a treated rabbit serum one attempt was made to obtain such information. Unfortunately, the serum employed was not as reactive as that used for the preceding experiment. The sol-gel mixture, treated as described in the foregoing, on incubation for 15 minutes gave but very slight effect; tested again at the end of ½, ¾, and 1 hour, it gave very severe shocks, but did not kill until after incubation for 1½ hours. The mixture was then centrifugated at 8000 revolutions for 1 hour, and the clear serum thus obtained was placed at 37 C. No

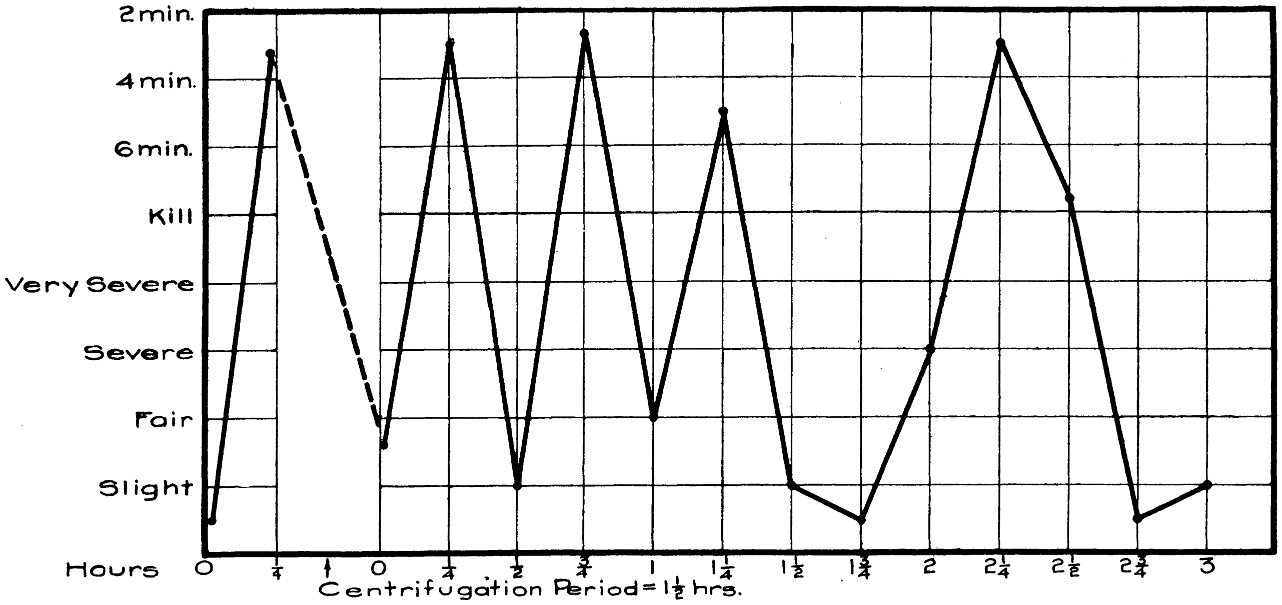

Chart 6. Centrifugation at 8000 r.p.m. of sol-gel rabbit-serum mixture No. 6, after incubation at 37 C. for 15 minutes (Table 35).

tests were made until the 12th hour; then it was tested at intervals of 15 minutes up to the 14th hour. The test at 12¼ hours killed in 3 minutes 50 seconds; of the other 7 tests only 1 was severe. The serum was allowed to remain at 37 C. and was again tested at quarter-hour periods, beginning at 24 hours. These tests gave but slight effects. In view of the fact that but 1 of 8 tests made at 12 hours was fatal, it is quite likely that a similar result might have been obtained after 24 hours.

This experiment, such as it is, shows that the toxicity may persist at 37 C. for 12 hours. With a serum of greater toxicity the result might have been more marked.

EFFECT OF ALKALI AND ACID ON TOXICITY

The only observations which have been made on the effect of alkali and acids on guinea-pig anaphylatoxin are those of Friedberger and Moreschi.[4] They found that the addition of 0.1 c.c. of N NaOH to 3.5 c.c. of anaphylatoxin, the latter representing lethal doses of 1.5 c.c., did not destroy the poison after a contact of 6 minutes, whereas such contact for 2 hours at room temperature apparently did do so. The addition of 0.5 c.c. of N NaOH seemed to destroy it in 6 minutes as well as in 2 hours. The animals which survived the injection of the alkalinized serum died, however, within 24 hours. On the other hand, the addition of corresponding amounts of N HCl and contact for 2 hours apparently had no effect on the toxicity. The additions mentioned correspond to 0.0286 and 0.143 c.c. normal solution per cubic centimeter of serum.

In view of the importance of these results it was desirable to make a more extended study of the effects of acid and alkali not only upon the poison, but also upon the production of anaphylatoxin. This work was carried out with rabbit serum, tho it would have been better to have made similar experiments with rat serum.

Before beginning this work the alkalinity of several normal rabbit sera was approximately determined by titration with N/20 HCl, using a sensitive litmus paper as an indicator. For this purpose 5 c.c. of the serum were placed in a large evaporating dish, distilled water added, and the whole warmed to 40 C. before adding the acid. The neutral point was apparently reached by the addition of 0.024 c.c. normal acid per cubic centimeter of rabbit serum. The addition of 0.03 c.c. gave a distinct acidity. The sera of fasting rabbits apparently required a trifle more acid, namely, 0.0265 to 0.027 c.c.; this alkalinity was not increased by heating such serum at 60 C. for ½ to 1 hour, but the heating seemed to make the protein more coagulable by the acid. Tested in the same way, a guinea-pig serum required 0.023 c.c., while a rat serum took 0.027 c.c. normal acid per cubic centimeter of serum. It would seem therefore that the slight differences in the alkalinity of these three kinds of sera would hardly account for their variability as regards anaphylatoxin-production. It may be added that, after treatment with agar, the rabbit serum appears to show a lessened alkalinity (0.019 to 0.021 c.c.).

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[4] Berl. klin. Wchnschr., 1912, 49, p. 743.

For the following experiments, sol-gel mixtures (No. 6) were prepared in the usual way, then placed in cracked ice for 2 hours. A small portion was then removed, centrifugated, and tested at once; the balance was placed at 37 C., and like portions were removed and similarly treated at intervals of 15 minutes. In all tests, the equivalent of 3 c.c. of serum was injected.

For each experiment a single rabbit serum, previously warmed to 37 C., was divided into 2 portions of 27 c.c. each. To one of these was added the desired amount of normal Na_2CO_3 or HCl, and to the other portion, serving as a control, a corresponding amount of distilled water. The two portions were then treated with the 37 C. sol, and eventually tested as indicated. It will be noted, therefore, that for each experiment there is a corresponding control. The two portions were incubated at the same time, each control being injected about a minute after the regular test animal. It would have been preferable if one pooled serum had been used for all three of the following experiments, but this, at the time, seemed impracticable.

For the first experiment (Table 36), the normal alkalinity was tripled by the addition of 0.048 c.c. normal Na_2CO_3 per cubic centimeter of serum. It will be noted that there is no evidence of poison-production in such alkaline serum, tho the duration of the experiment was 2 hours; the corresponding control series gave 2 fatal shocks. An excess of alkali appears to inhibit the generation of the anaphylatoxin. This is in accord, as it were, with the findings of Friedberger and Moreschi that alkali destroys the ready-made anaphylatoxin. This experiment is deserving of repetition with the more reactive rat serum.

TABLE 36

Production of Anaphylatoxin in Rabbit Serum of Triple Alkalinity (Sol-Gel Mixture 6. Ratio 0.1:1. Dose 3 c.c.)

0.024 c.c. N Na_2CO_3 per c.c. Serum				Control with Same Serum			
Guinea-Pig	Weight	Hours at 37 C.	Result	Guinea-Pig	Weight	Hours at 37 C.	Result
1	208	0	Nil	1	199	0	4′20″
2	196	¼	Slight	2	209	¼	Nil
3	210	½	Very slight	3	205	½	3′
4	176	¾	" "	4	198	¾	Nil
5	181	1	Slight	5	183	1	Slight
6	211	1¼	Very slight	6	209	1¼	Very slight
7	207	1½	Slight	7	195	1½	Nil
8	199	1¾	Very slight	8	188	1¾	"
9	177	2	" "	9	187	2	Slight

In the second experiment (Table 37), the alkalinity was doubled by the addition of 0.024 c.c. N Na_2CO_3 per cubic centimeter of serum, with the surprising result that the toxicity was decidedly increased as compared with that of the corresponding control. Unless this is an error incidental to the variable resistance of guinea-pigs, it would indicate that a moderate increase in the alkalinity favors the production of anaphylatoxin. It should be noted, however, that Friedberger and Moreschi employing a trifle more alkali (0.0286 c.c. N NaOH), as compared with the foregoing amount, destroyed the ready-made anaphylatoxin after contact for 2 hours at room temperature.

TABLE 37

PRODUCTION OF ANAPHYLATOXIN IN RABBIT SERUM OF DOUBLE ALKALINITY (SOL-GEL MIXTURE 6. RATIO 0.1:1. DOSE 3 C.C.)

0.024 c.c. N Na_2CO_3 per c.c. Serum				Control with Same Serum			
Guinea-Pig	Weight	Hours at 37 C.	Result	Guinea-Pig	Weight	Hours at 37 C.	Result
1	204	0	Slight	1	187	0	Very slight
2	170	¼	Very slight	2	175	¼	Very slight
3	206	½	9′45″	3	205	½	Nil
4	190	¾	5′50″	4	209	¾	Very slight
5	171	1	Very slight	5	176	1	3′50″
6	183	1¼	Very slight	6	208	1¼	Very slight
7	188	1½	Near-kill	7	205	1½	Slight
8	174	1¾	Nil	8	195	1¾	Very slight
9	207	2	3′25″	9	173	2	Slight

In the third experiment (Table 38) the serum was rendered sesqui-alkaline by the addition of 0.012 c.c. N Na_2CO_3 per cubic centimeter of serum. Here the increased anaphylatoxin-production was even more striking than in the preceding since every test made during the 2 hours' experiment gave either a very severe shock or a kill. The control tests, while not so severe, showed that the serum used was more reactive than those employed in the two preceding experiments. Further tests of sesqui-alkaline serum are desirable in view of these results.

TABLE 38

PRODUCTION OF ANAPHYLATOXIN IN RABBIT SERUM OF SESQUI-ALKALINITY (SOL-GEL MIXTURE 6. RATIO 0.1:1. DOSE 3 C.C.)

0.012 c.c. N Na_2CO_3 per c.c. Serum				Control with Same Serum			
Guinea-Pig	Weight	Hours at 37 C.	Result	Guinea-Pig	Weight	Hours at 37 C.	Result
1	195	0	3′5″	1	201	0	Nil
2	195	¼	Very severe	2	175	¼	Near-kill
3	171	½	Very severe	3	170	½	4′
4	180	¾	Very severe	4	202	¾	Moderate
5	199	1	3′45″	5	203	1	Nil
6	206	1¼	3′10″	6	210	1¼	Nil
7	205	1½	Very severe	7	209	1½	5′
8	182	1¾	3′5″	8	170	1¾	Fair
9	175	2	Near-kill	9	171	2	3′10″

In the fourth experiment (Table 39) the serum was rendered semineutral by the addition of 0.012 c.c. N HCl per cubic centimeter of serum. In this set there is a marked agreement with the control, showing that a slight decrease in the alkalinity is without effect on the production of anaphylatoxin.

TABLE 39

Production of Anaphylatoxin in Rabbit Serum, Semineutral (Sol-Gel Mixture 6. Ratio 0.1:1. Dose 3 c.c.)

0.012 c.c. N HCl per c.c. Serum				Control with Same Serum			
Guinea-Pig	Weight	Hours at 37 C.	Result	Guinea-Pig	Weight	Hours at 37 C.	Result
1	193	0	Slight	1	187	0	Slight
2	198	¼	Very slight	2	182	¼	Very slight
3	171	½	Moderate	3	173	½	Very slight
4	186	¾	3'40"	4	181	¾	3'15"
5	201	1	Nil	5	170	1	Severe
6	187	1¼	Severe	6	172	1¼	Moderate
7	179	1½	4'	7	184	1½	3'15"
8	170	1¾	Very severe	8	172	1¾	Moderate

In the fifth experiment (Table 40) the serum was very faintly acidified by the addition of 0.03 c.c. of N HCl per cubic centimeter of serum. This series of tests was discontinued at the end of 1¼ hours, and up to the end of that time the results indicated a decrease, if not suppression, of anaphylatoxin-production. The experiment should be repeated and extended, especially since the control showed but a moderate reactivity; the only fatal result, that in the immediate test made after icing (No. 1), may well be due to the use of a very susceptible guinea-pig. In view of the results of Friedberger and Moreschi, it might be expected that acid would have no influence on the poison-production.

TABLE 40

Production of Anaphylatoxin in Rabbit Serum, Faintly Acidified (Sol-Gel Mixture 6. Ratio 0.1:1. Dose 3 c.c.)

0.03 c.c. N HCl per c.c. Serum				Control with Same Serum			
Guinea-Pig	Weight	Hours at 37 C.	Result	Guinea-Pig	Weight	Hours at 37 C.	Result
1	172	0	Slight	1	181	0	3'45"
2	205	¼	Very slight	2	207	¼	Slight
3	176	½	Slight	3	202	½	Moderate
4	185	¾	Very slight	4	172	¾	Fair
5	195	1	Very slight	5	171	1	Slight
6	206	1¼	Moderate	6	200	1¼	Very slight

In a sixth experiment (Table 41) the conditions were changed; the object being to ascertain the effect produced by an excess of acid and alkali when allowed to react with sera for 2 hours at 37 C. The serum was divided into 2 portions; one received 0.048 c.c. N HCl per cubic centimeter, while the other received 0.048 c.c. N Na_2CO_3 per cubic centimeter. After these mixtures had been incubated for 2 hours at 37 C., they were adjusted to approximately the original reaction by the addition of 0.048 c.c. of normal alkali to the former, and the same amount of normal acid to the latter. They were then treated by the sol-gel method in exactly the same way as in the preceding experiments. It is significant that on incubation these sol-gel mixtures did not give the usual precipitate, behaving in this respect like inactivated serum (Bordet).

The results in this experiment were striking, tho unfortunately no parallel control tests were made with the same serum untreated with chemicals. As seen in the table, the acidified serum showed practically no evidence of poison-production up to 2 hours, the duration of the experiment. The alkalinized serum showed the same behavior, but the last, or 2-hour, test did cause acute death. Whether this means that the poison was beginning to form at that time, or that by chance a very susceptible guinea-pig was then used, cannot be determined without further tests.

It would appear from this experiment that a serum is denatured by previous treatment with acid or alkali, the former perhaps being more active than the latter. The results of the acid treatment seem to confirm those of the preceding experiment. Similarly, the effects with alkali-treated serum correspond with those obtained in the first experiment (Table 36), where the same amount of alkali was used in the presence of the sol-gel.

TABLE 41

PRODUCTION OF ANAPHYLATOXIN IN RABBIT SERUM TREATED WITH ACID (OR ALKALI) FOR 2 HOURS AT 37 C. BEFORE ADDITION OF AGAR (SOL-GEL MIXTURE 6. RATIO 0.1:1. DOSE 3 c.c.)

0.048 c.c. N HCl per c.c. Serum				0.048 c.c. N Na_2CO_3 per c.c. Serum			
Guinea-Pig	Weight	Hours at 37 C.	Result	Guinea-Pig	Weight	Hours at 37 C.	Result
1	210	0	Very slight	1	205	0	Very slight
2	208	¼	Very slight	2	202	¼	" "
3	198	½	Nil	3	203	½	" "
4	186	¾	Nil	4	183	¾	" "
5	180	1	Very slight	5	175	1	" "
6	205	1¼	Slight	6	170	1¼	" "
7	207	1½	Slight	7	172	1½	Nil
8	209	1¾	Very slight	8	183	1¾	Very slight
9	211	2	Very slight	9	177	2	2′50″

The general conclusions to be drawn from these experiments are: first, that an excess of alkali (0.048 c.c. per cubic centimeter) alters the serum and prevents or greatly retards the anaphylatoxin-production; second, a moderate increase in alkalinity may actually favor its production; third, a slight decrease in alkalinity is without appreciable effect; and fourth, a faint acidity may retard, while an excess of acid (0.048 c.c. per cubic centimeter) may alter the serum so that poison-production is suppressed. An excess of acid as well as of alkali therefore interferes with the production of anaphylatoxin.

Action of Acid on Anaphylatoxin.—The effects observed when an excess of acid or of alkali was added to the agar-serum mixtures might be interpreted as due to a weakening or destruction of the hypothetical ferment. It is more probable, however, that the very labile matrix is involved in the reaction. The poison once formed may be looked upon as relatively stabile, a fact which is indicated by its behavior under the application of heat at 60 C. or 70 C. Since it had been shown that the toxicity of a treated serum persists after the removal of the agar (Table 35), it seemed desirable to test the action of acid and of alkali on such anaphylatoxin repeating, as it were, the work of Friedberger and Moreschi with guinea-pig anaphylatoxin.

For the following experiment (Table 42) a serum (68 c.c.) was treated by the sol-gel method, the procedure being exactly the same as that used for the experiments of Table 35. After the first fatal shock had been obtained (No. 3), the mixture was centrifugated at 8000 revolutions for 1 hour. The clear serum when tested at once was fatal (No. 4). It was then placed at 37 C. and after the next fatal shock (No. 6) it was divided into 2 portions: to one portion, 0.072 c.c. N HCl per cubic centimeter of serum was added, while the other portion served as a control. Both were then returned to the water-bath at 37 C. and tested at intervals of 15 minutes. The equivalent of 3 c.c. serum was used as the dose, and before injection this was neutralized by the addition of 0.3 c.c. N Na_2CO_3. The tests of control and corresponding treated serum were made together at each test period.

The results are given in Table 42 and shown also in Chart 7. It will be observed that the control tests (Nos. 7 to 12) show persistence of the poison, tho one-half of the animals show very little effect, because of individual resistance. The tests with acid-treated serum (Nos. *7a* to *14a*) seem to show a marked drop in toxicity, since but 1 of 8 proved fatal and the others were all weak. It would appear reasonable to assume from this experiment that some of the poison had been destroyed by the acid, but this cannot be considered as established without further trials.

TABLE 42

BEHAVIOR OF ANAPHYLATOXIN TOWARD N HCL (0.072 C.C. PER C.C. SERUM)

Control: Portion Untreated with Acid					Portion Treated with Acid			
Guinea-Pig	Weight	B. C. at 37 C. (min.)	A. C. at 37 C. (hr.)	Result	Guinea-Pig	Weight	With Acid at 37 C. (hr.)	Result
1	187	0		Very slight				
2	175	15		Nil				
3	189	30		3′40″	← Mixture centrifugated at 8000 r.p.m. for 60′			
4	181		0	4′50″				
5	170		¼	Slight				
6	198		½	3′50″	← Mixture divided, one-half treated with acid			
7	200		¾	Very slight	7a	188	¼	Moderate
8	175		1	4′5″	8a	182	½	Slight
9	205		1¼	Very slight	9a	201	¾	Very slight
10	189		1½	4′	10a	175	1	Nil
11	182		1¾	5′10″	11a	170	1¼	Very slight
12	198		2	Nil	12a	201	1½	3′55″
13			2¼		13a	171	1¾	Slight
14			2½		14a	207	2	Very slight

The foregoing experiment was repeated with this difference, that as soon as the sol-gel mixture 6 became toxic, which occurred after incubation for 15 minutes, it was divided into 2 portions: to one portion, 0.072 c.c. N HCl per cubic centimeter of serum was added; the other portion was retained as a control. Both portions were then centrifugated at 8000 revolutions for 15 minutes, and then tested at once, the balance of each portion being placed at 37 C. and retested at intervals of 15 minutes. As before, the equivalent of 3 c.c. of serum was used as the test dose. In the case of the acid-treated serum, this was neutralized by the addition of 0.3 c.c. N Na_2CO_3 before injection.

TABLE 43

BEHAVIOR OF ANAPHYLATOXIN TO N HCL (0.072 C.C. PER C.C. SERUM)

Control: Portion Untreated with Acid					Portion Treated with Acid			
Guinea-Pig	Weight	B. C. at 37 C. (min.)	A. C. at 37 C. (hr.)	Result	Guinea-Pig	Weight	With Acid at 37 C. (hr.)	Result
1	210	0		Moderate				
2	176	15		27′	← Mixture divided; acid added and both centrifugated at 8000 r.p.m. for 15′			
3	180		0	Slight	3a	185	0	3′40″
4	170		¼	3′30″	4a	176	¼	Near-kill
5	210		½	3′20″	5a	195	½	Slight
6	207		¾	Very slight	6a	180	¾	Slight
7	196		1	Very slight	7a	206	1	Slight
8	174		1¼	Very severe	8a	203	1¼	Near-kill
9	172		1½	Slight	9a	202	1½	4′20″
10	185		1¾	Very slight	10a	171	1¾	4′ 5″
11			2		11a	209	2	Moderate

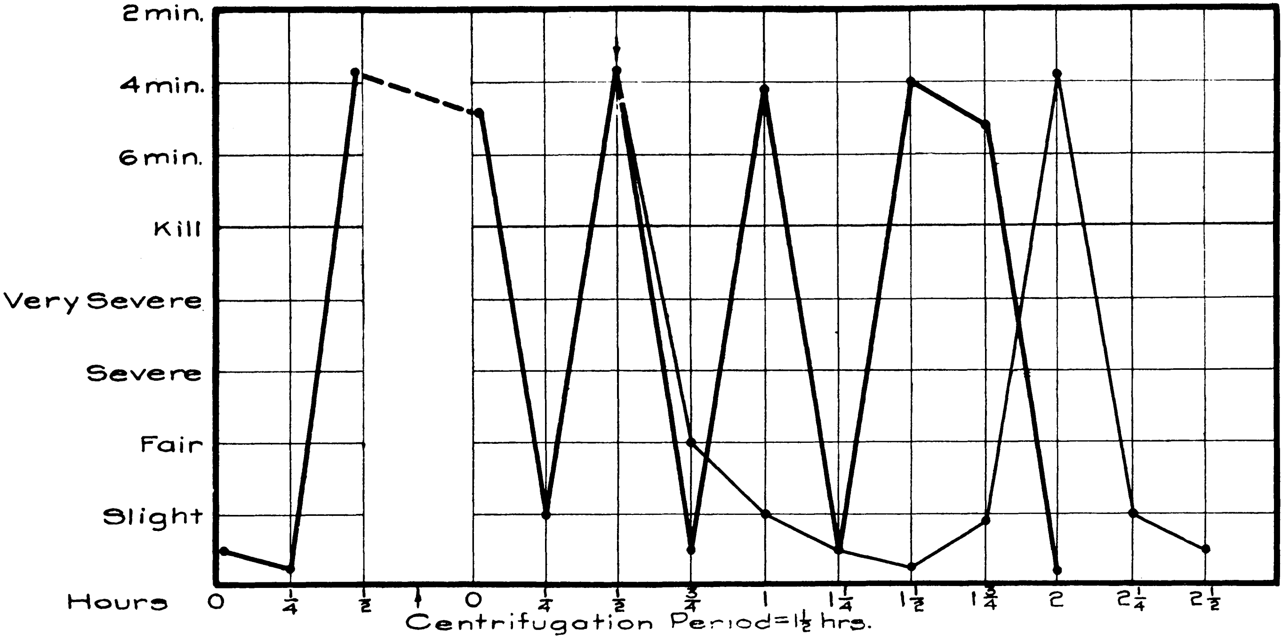

Chart 7. Behavior of anaphylatoxin to N HCl; the lighter line is the acid curve, the heavy one is that of the untreated portion (Table 42).

The results of these tests are shown in Table 43. It will be seen that the toxicity of the acid-treated serum (*3a* to *11a*) is fully as great, if not more so, than that of the control portion (Nos. 3 to 10). While it is probable that some adsorption of the acid by the agar took place, it is unlikely that this occurred to any great extent in view of the short contact before centrifugation. It must be concluded from this experiment that the anaphylatoxin is not affected by the acid in the concentration given.

PRODUCTION OF ANAPHYLATOXIN AT 50 C.

Since the agar-serum mixture does not become toxic when kept in ice, but does become so when incubated at 37 C., it seemed as if at a still higher temperature the poison-production might be more marked and also more rapid. This certainly could be expected if dispersion was the sole factor in the reaction, provided, however, that such increased temperature did not induce changes in the dispersed colloid.

The first experiment made with this object in view ended in a wholly negative result. A sol-gel mixture (No. 6) was prepared in the usual way, and after being kept at 0 C. for 2 hours was placed in a Roux water bath at 50 C. Nine consecutive tests, made at intervals of 15 minutes, up to 2 hours, with the equivalent of 3 c.c. of serum as the test dose, had practically no effect. The conclusion seemed justified that at this temperature, the poison-production could not take place. However, since this experiment was not accompanied by a parallel control, with a portion of the same serum kept at 37 C., the result was open to doubt. Accordingly, the experiment was repeated, provision this time being made for a rigid control.

The sol-gel mixture was prepared as before, and placed for 2 hours at 0 C. A portion, the equivalent of 3 c.c. of serum, was then removed, centrifugated, and tested (No. 1); the remainder was in turn divided into 2 portions, one of which was placed at 37 C., and the other at 50 C. At intervals of 15 minutes, portions were removed from each of these and tested as before. The parallel tests were made within 2 or 3 minutes of each other. As will be seen from Table 44, the two portions (Tests 2 to 4 and 2a to 5a) behaved exactly alike. After Test 5a was made, the mixture kept at 50 C. was in turn divided into 2 parts; one remained at 50 C., and the other at 37 C. Both portions were tested, as before, at intervals of 15 minutes; they again showed a marked agreement in results.

Apparently, anaphylatoxin can be produced by the incubation of a sol-gel mixture at 50 C., and about as well as at 37 C. This result corresponds to the production of toxicity in normal rabbit blood which is allowed to clot at 50-60 C.

There can be no doubt but that the portion which was placed at 50 C. developed anaphylatoxin, but it may be questioned whether this poison was produced at that temperature. The iced agar-serum mixture when placed in the water bath, necessarily warmed up slowly, and it is more than probable that the poison was produced before the mixture was heated up to 50 C. The control test 2 shows that the poison was produced at 37 C. within 15 minutes; to raise the temperature of the iced mixture to 50 C. may require from 5 to 8 minutes, and this time is sufficient to make the poison, especially as the optimal temperature is probably about 45 C.

In view of these considerations it is not surprising to find that iced agar-serum mixtures when placed at 55 C., or even at 60 C., likewise develop anaphylatoxin. Here also it is to be understood that the poison is produced en route, as it were, before the temperature reaches or passes 50 C.

When a rabbit serum is dialyzed in a collodium sac against distilled water at 50 C. for a quarter to a half hour, and is then treated by the sol-gel method and the mixture placed at 37 C., it will be found that very little poison is produced. It follows that even a short exposure of the serum at 50 C. causes some change or modification in the matrix of the poison. This effect is more marked when the dialysis takes place at 55 C. The results are most conclusive when the more reactive rat serum is employed for such tests. Experiments with this serum will be considered in Part IV.

TABLE 44

Production of Anaphylatoxin in Rabbit Serum at 50 C.

Portion at 37 C.				Portion at 50 C.			
Guinea-Pig	Weight	Hours at 37 C.	Result	Guinea-Pig	Weight	Hours at 50 C.	Result
1	170	0	Very slight				
2	191	¼	5′40″	2a	172	¼	3′20″
3	171	½	Fair	3a	205	½	Moderate
4	188	¾	Very slight	4a	211	¾	Slight
5				5a	186	1	Very slight
6*	181	¼	5′25″	6a	176	1¼	2′45″
7	207	½	Very slight	7a	185	1½	Slight
8	187	¾	Severe	8a	193	1¾	Very slight
9	185	1	Near-kill	9a	197	2	Very slight
10	180	1¼	Slight	10a	173	2¼	Slight

* After Test 5a the 50 C. mixture was divided and one half placed at 37 C. (Nos. 6 to 10).

ATTEMPTED DEMONSTRATION OF FERMENT ACTION

Many attempts were made, similar to those employed in connection with the study of guinea-pig serum, to detect the presence of a ferment. The serial dilutions A and B of treated centrifugated sera with normal rabbit serum were prepared, and incubated at 37 C., after which they were tested at diverse periods (2 to 24 hours). Usually, little or no effect was obtained, but, at times, the portions tested at the 18- or 24-hour period caused death in the course of several hours. The test dose was 4 c.c., and this fact together with that of the prolonged incubation eventually led to the realization that such deaths were really due to the inherent primary toxicity of the serum.

It was hoped that better results would be obtained by diluting the treated centrifugated rabbit serum with normal rat serum. The fact that the latter is very readily toxifiable seemed to make it a reagent of choice for the detection of the ferment. When the dilutions A, B, and C were prepared with rat serum and incubated at 37 C. for from 2 to 5 hours and then tested, it was found that in dose of 1 c.c. they did produce acutely fatal shocks. Such results might be taken to indicate ferment action were it not for the fact that control tests demonstrated that they were due to the toxifying action of the residual agar, the trace left in the treated rabbit serum after centrifugation at 3000 revolutions. The presence of such residual agar was demonstrable, as in the case of guinea-pig serum, by recentrifugation at high speed.

Were a ferment concerned in the production of anaphylatoxin, it would be reasonable to expect that the toxicity would progressively increase in the agar-treated serum. It will be seen, however, by a study of the tables that the initial more or less rapid production of poison is not followed by an appreciable increase. Such being the case, it is hardly possible for the dilutions to give other results than those obtained. This method of approach has therefore failed to give any evidence of the participation of an enzyme in the production of anaphylatoxin.

RESISTANCE OF GUINEA-PIGS TO RABBIT ANAPHYLATOXIN

The apparent oscillation in the toxic effects, as noted in guinea-pigs after the injection of anaphylatoxin of diverse origin (including that of primarily toxic sera), was interpreted for a time as indicating a periodicity or wave formation of the poison. This view implying a variability in the poison and a uniformity in the resistance of the guinea-pig was definitely abandoned as the result of an extended series of tests made with rabbit anaphylatoxin. It was found that the resistance of the guinea-pig to the poison varies considerably, and that the production of little or no effect in an animal does not necessarily imply the absence of anaphylatoxin. This fact became apparent when, in making serial tests at one-quarter hour intervals, 2 guinea-pigs of the same weight were injected each time with the same material; the two injections being made within half a minute of each other. In all the tests referred to in the following, the agar rabbit anaphylatoxin was used in dose of 3 c.c.

An analysis of 110 pairs of such tests, showed that in 55 pairs (50%) the effects were exactly alike, the two individuals of each paired test responding in the same manner; that is, 2 acute deaths, or 2 severe shocks, or 2 nil effects.

In 27 pairs the reaction was nearly alike, the 2 animals of each double test showing some difference, but not marked. Thus, of 2 guinea-pigs receiving the same dose at the same time, one might die of acute shock and the other might experience a very severe shock or near-kill. Such variation in individuality was to be expected.

On the other hand, a third group of 28 pairs of tests (25%) showed, rather unexpectedly, an extreme variation. This group includes those paired tests in which one animal dies, or receives a very severe shock, while its mate shows little or no effect. The results obtained in 8 pairs of such tests taken at random, are given in Table 45. For other inoculations in pairs, see Tables 55 and 56.

TABLE 45

SHOWING EXTREME INDIVIDUAL RESISTANCE TO SIMULTANEOUS INJECTION OF THE SAME DOSE OF ANAPHYLATOXIN IN PAIRED GUINEA-PIGS

Guinea-Pig		Result
No.	Weight	
1	200	3′25″
1a	201	Very slight
2	187	Slight
2a	186	6′5″
3	205	4′40″
3a	204	Slight
4	190	2′40″
4a	188	Nil
5	194	Slight
5a	196	3′50″
6	198	2′ 5″
6a	198	Very slight
7	195	Slight
7a	197	3′17″
8	185	Slight
8a	185	2′35″

In view of these results it is evident that one cannot speak of a strictly minimal lethal dose. The amount of poison necessary to produce a fatal shock in every one of a series of 8 or 10 animals is probably at least twice that which gives an occasional kill. Furthermore, great care must be taken in drawing conclusions from serial tests in which but 1 or 2 die of 8 or 10 animals. A death or severe shock does not necessarily mean an increase in the amount of the poison, and per contra the absence of a severe shock does not mean that the poison is not present. And again, it is obviously wrong to assume (as has often been done in the past) that the poison has been destroyed when a single test (or even a number of single tests) happens to be negative. In such cases, a multiple dose and duplicate tests are essential to avoid error.

SUMMARY

The production of anaphylatoxin in normal rabbit serum is not as facile as in guinea-pig or rat serum.

The individual sera show considerable difference in the ease with which they yield the poison. The presence of lipoidal matter is not inhibitive. The sera after a short fast appear to be more reactive. A varied diet appears to be favorable to the reaction.

The primary toxicity of the normal serum is greatly influenced by the method of defibrination as well as by the state of the animal. The sera obtained by rod defibrination may be toxic in dose of 6 c.c. The sera from cachectic treated rabbits may cause acute fatal shock in dose of 0.05 c.c., which represents a toxicity 120 times greater than that of normal serum.

Normal rabbit serum can be toxified with agar so that 3 c.c. will be fatal.

Attempts at toxifying with agar sol (50 C.), or gel (0 C.), were less favorable than like experiments with guinea-pig serum.

The sol-gel method appears to be the best means of inducing poison-production. Mixture 3, which contains but 0.025 mg. of agar per cubic centimeter of serum (1:40000), can become fatally toxic. The optimal mixture is probably No. 6. An excess of agar appears to have an unfavorable action.

The poison is not produced at 0 C.; incubation at about 37 C. is necessary. The apparent production of anaphylatoxin in agar-serum mixtures incubated at 50 C. or higher really occurs before such high temperature is reached.

The speed of poison-production is considerable, suitable sera being toxified in less than 15 minutes. The poison once developed persists in the serum even after complete removal of the agar by high-speed centrifugation. In such serum, the toxicity may persist at 37 C. for more than 12 hours.

An excess of acid as well as of alkali interferes with the production of anaphylatoxin. A moderate increase in the alkalinity may increase the anaphylatoxin-production. The ready-made anaphylatoxin is not affected by a moderate acidity.

Dialysis of rabbit serum in collodium sacs at 50 C. renders it less toxifiable. This fact as well as the behavior with excess of acid or alkali indicates that the matrix of the poison is labile.

Attempts to demonstrate the participation of a ferment in the reaction failed.

Marked variations in the susceptibility of guinea-pigs to the rabbit anaphylatoxin are possible. This fact explains the apparent oscillation in the toxicity of normal as well as of treated sera.